Synthesis of well-defined phosphate-methylated DNA fragments: the application of potassium carbonate in methanol as deprotecting reagent

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ABSTRACT

A new deprotection procedure in the synthesis of (partially) phosphate-methylated oligodeoxynucleotides has been developed, involving treatment of fully protected DNA fragments with methanolic potassium carbonate. It is shown that base deprotection can be accomplished in potassium carbonate/methanol without affecting the methyl phosphotriesters. This methodology enables us to synthesize, both in solution and on a solid support, DNA fragments which are phosphate-methylated at defined positions. The solid phase synthesis, however, turns out to be accompanied by considerable demethylation of the phosphotriesters. It is demonstrated that this demethylation does not occur during the deprotection or work-up procedure. Furthermore, it was found that the latter side-reaction is suppressed when the standard capping procedure with acetic anhydride is included.

INTRODUCTION

The past several years have seen a growing interest in various backbone-modified DNA analogues. Especially, the specific interactions of modified DNA fragments with proteins and the selective inhibition of gene expression with synthetic antisense oligodeoxynucleotides have received much attention.[1-4] Several classes of phosphate-modified oligodeoxynucleotides have been chemically synthesized, including nucleotides containing phosphorothioates[5,6], methylphosphonates[7,8] and alkyl phosphotriesters.[9-12] An interesting series in the last class concerns phosphate-methylated DNA analogues, which were already introduced in 1971 by Miller *et al.*[12] It was shown that, in comparison to natural fragments, phosphate-methylated oligodeoxynucleotides exhibit enhanced hybridization with complementary nucleotides.[12,13] Moreover, it was demonstrated that both R_P and S_P configurations of methyl phosphotriesters can be accommodated in the B DNA double helix[13], in contrast to other oligo-alkyl phosphotriesters (e.g. ethyl, isopropyl).[14,15] Recently, in biological experiments Moody *et al.* showed that phosphate-methylated DNA oligomers can block gene expression in a sequence-specific manner.[16,17]

Nowadays, the preparation of several types of backbone-modified oligodeoxynucleotides has been enormously improved by the development of automated solid phase procedures. In particular, oligodeoxynucleotides containing phosphorothioates and methylphosphonates can be prepared by adapting the phosphite triester method[18] (based on phosphoramidite chemistry).[19-22] A solid phase synthesis of phosphate-methylated DNA fragments, however, can not be performed by simply applying commercially available methyl phosphoramidites, because base (A, C and G) deprotection in concentrated aqueous ammonia will inevitably be attended by simultaneous deprotection of the methyl phosphotriesters.

In this paper, we wish to report a new procedure for the synthesis of completely or partially phosphate-methylated DNA structures, which is also applicable for solid phase methods. This approach involves, amongst others, the application of the 9-fluorenylmethoxycarbonyl (Fmoc) group as base protecting group,[23] the β-cyanoethyl group as phosphate protecting group[24] and potassium carbonate in methanol as deprotecting reagent. It is demonstrated on synthetic model compounds that methanolic potassium carbonate can effectively cleave off the Fmoc group without affecting the methyl phosphotriesters. The first trials of this concept in solid phase synthesis were successful, although it was observed that part of the methyl phosphotriesters was demethylated. We show that this demethylation can not be ascribed to side-reactions during the deprotection and work-up procedure, but instead, occurs during the solid phase synthesis itself.

RESULTS AND DISCUSSION

A successful solid phase procedure for phosphate-methylated DNA requires at least a base protecting group which can be removed under conditions that do not affect the methyl phosphotriesters. Preferably, under the same deprotection conditions the ester bond linking the DNA to the support has to be cleaved as well. Recently, 9-fluorenylmethoxycarbonyl

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(Fmoc) was introduced for the protection of the nucleotide bases in the solution synthesis of phosphate-methylated dinucleotides.[25] In that study, it was shown that the Fmoc group could be effectively deblocked by reacting the fully protected dimers with excess triethylamine in pyridine.

Based on the Fmoc protection strategy a synthesis of longer phosphate-methylated DNA oligomers has been described,[16] starting from natural (charged) DNA fragments. First, DNA fragments with the desired sequence are prepared on a solid support using the conventional β-cyanoethylamidite procedure.

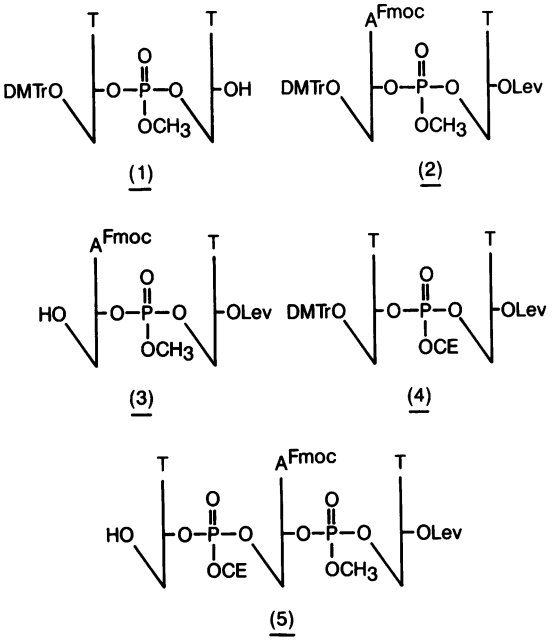


Figure 1: Structures of compounds used in trial deprotection experiments.

Then, the base-amino functions and the terminal hydroxyl groups of these fragments are being protected with a Fmoc group, after which the methyl phosphotriesters are introduced in a mixture of N, N-dimethylformamide, 2,6-lutidine and methanol in the presence of p-toluenesulphonylchloride.[12] Finally, the base-amino and hydroxyl functions are deprotected by treatment of the protected DNA nucleotide with a 1:1 (v/v) mixture of triethylamine and pyridine during several hours.

Unfortunately most of the chemical steps of the above mentioned synthetic method for the preparation of phosphate-methylated DNA are poorly defined with regard to degree of conversion and side-reactions. Additionally, during removal of the Fmoc protective group in a 1:1 (v/v) mixture of triethylamine and pyridine some methyl phosphotriesters may be deprotected as well. Under the latter reaction conditions the phosphate-methylated dimer **1** showed, according to ^{31}P NMR (and TLC) experiments, a considerable degree of demethylation (14% in 6 hours). The ^{31}P NMR spectrum, after one day, revealed that more than 35% of the methyl phosphotriester was demethylated (Figure 2). Notwithstanding the fact that triethylamine appears to be acceptable as a deprotecting reagent for the solution synthesis of relatively short phosphate-methylated DNA oligonucleotides, considerable amounts of partially charged molecules will be formed during the deprotection of longer methylated DNA fragments.

Consequently, an improved deprotection method is required which is capable to cleave off the Fmoc group while leaving the methyl phosphotriesters intact. First, we tested some mild nitrogen bases as deprotecting reagent. The Fmoc-protected nucleotides 5'-O-DMTr-dA-Fmoc, 5'-O-DMTr-dC-Fmoc and 5'-O-DMTr-dG-Fmoc (**9a**–**9c**)[26] were chosen as test systems for the amino group deprotection (TLC experiments), while the dimer **1** served as a model compound to investigate the stability of the phosphotriester (TLC and ^{31}P NMR experiments). It was found that treatment with morpholine (10% in acetonitrile) brings about a fast base deprotection (approx. 1 hour). Unfortunately,

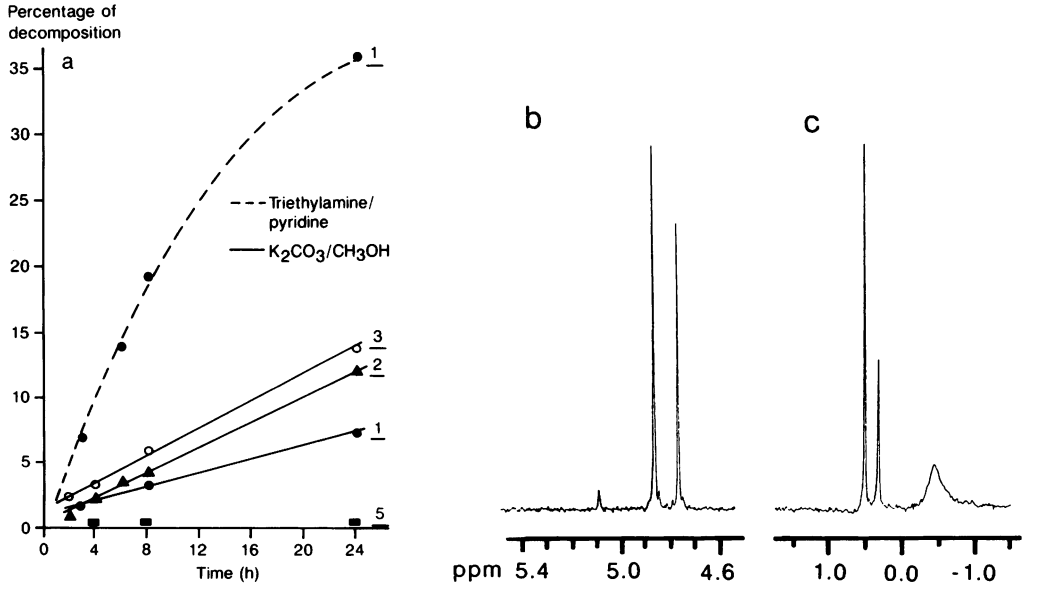


Figure 2: Stability of the methyl phosphotriester as determined via ^{31}P NMR measurements. (a): Percentage of demethylation in dimer **1** (8 μmol) in triethylamine (200 μl)/pyridine (100 μl)/pyridine-d_5 (100 μl) (---) and percentage of chain cleavage in dimers **1**, **2**, **3** and trimer **5** (8 μmol) in 0.05 M potassium carbonate/methanol (350 μl) and acetonitrile-d_3 (50 μl) (-). (b): ^{31}P NMR spectrum of dimer **1** in potassium carbonate/methanol after 24 hours, the demethylated dimer **1** would resonate at 3.84 ppm but was not observed. (c): ^{31}P NMR spectrum of dimer **1** in pyridine/triethylamine after 24 hours; the differences in chemical shifts of the major resonances observed in spectra (b) and (c) have to be attributed to the use of different solvent systems (see experimental section for further details).

also under these conditions a rapid demethylation of the phosphotriester occurs. ^{31}P NMR analysis revealed the formation of 12% of demethylated dimer in 1 hour. Recently, another group described that 50% demethylation occurs after 3 hours when a similar dimer is treated with piperidine/acetonitrile (1:9 v/v).[27] The less nucleophilic base diisopropylethylamine (10% in acetonitrile) cleaves the Fmoc group very slowly and therefore appears not suitable as a deprotecting reagent.

We turned our attention to other bases, because all amines that deprotect the Fmoc group are sufficiently nucleophilic to deblock the methyl groups of the phosphotriesters (attack at carbon). However, a hard base like hydroxylate or fluoride ion will cause demethylation as well, due to hydrolysis of the phosphotriester (attack at phosphorus). We reasoned that a compromise might be found by using 'mild methanolate' as Fmoc deprotecting reagent. Indeed, a 0.05 Molar solution of potassium carbonate in dry methanol appeared to be able to deblock the Fmoc-protected 5'-O-DMTr-nucleosides in a reaction time comparable to that of a deprotection in a mixture of triethylamine and pyridine (i.e. 3–4 hours). However, acting as a hard nucleophile, methanolate might also cause some transesterification on the phosphotriester, leading either to an exchange of the methoxy group at phosphorus or, at the worst case, to chain cleavage of the phosphate-methylated DNA molecule. When the dimer **1** was subjected to potassium carbonate in methanol, ^{31}P NMR experiments revealed that after 6 hours less than 3% of the dinucleotide was cleaved (Figure 2).

The suitability of potassium carbonate in methanol as deprotecting reagent was further investigated by treatment of the dinucleotide **2**. TLC and ^{31}P NMR monitoring of this experiment showed that the Fmoc group was completely removed within 3 hours, while only a slight percentage of chain cleavage (3–4%) could be detected. Furthermore, it was observed that the levulinoyl ester was saponified within 10 minutes. Similar results were obtained upon exposure of the phosphate-methylated dimer **3**, bearing a free 5'-hydroxyl group, to the methanolic potassium carbonate solution. In Figure 3 the conversion of the Fmoc-protected dimer **3** into derivative **3b**, its deprotected analogue, is depicted. Compounds **3a** and **3b** show two peaks in the ^{31}P NMR spectrum originating from their R_P and S_P diastereoisomers. According to ^{31}P NMR analysis, the Fmoc cleavage was complete in 2 hours (Figure 3), while no starting material could be detected by TLC after 4 hours. In this time the dinucleotide cleavage remained limited to 3% (Figure 2). The similar behaviour of **2** and **3** in methanolic potassium carbonate indicates that no detectable neighbouring group participation of the free 5'-hydroxyl group occurred (leading to phosphotriester cleavage or 5'-5' bond formation).

In contrast to triethylamine, potassium carbonate in methanol will hydrolyse the ester bond linking DNA oligomers to solid supports. For this reason, we felt that it should be possible to develop a solid phase synthesis of phosphate-methylated DNA using this new deprotection method. Furthermore, we expected that methanolic potassium carbonate would accomplish deprotection of a cyanoethyl-blocked phosphate group as well, so that well-defined partially phosphate-methylated DNA fragments may be synthesized. Indeed, upon treatment of compound **4** with potassium carbonate in methanol the β-cyanoethyl group was almost instantaneously removed (TLC: $R_f \rightarrow 0$; ^{31}P NMR). In this experiment it was also observed that the levulinoyl ester was saponified. However, this reaction lasted markedly longer than in case of the neutral dimer **2**, namely 4 hours instead of 10 minutes.

The protected trimer **5** is a model compound uniting all functionalities relevant in the solid phase procedure. This

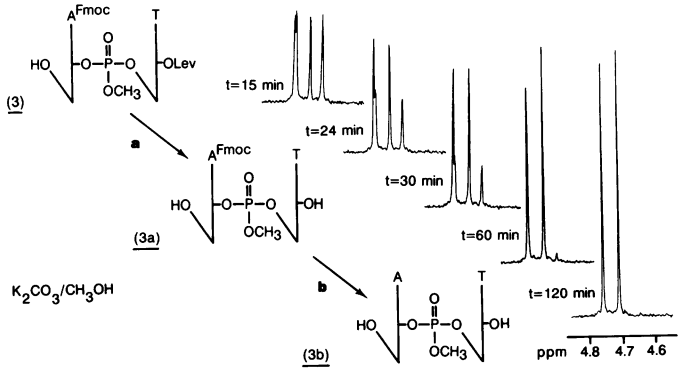


Figure 3: Fmoc deprotection (step **b**) of dimer **3** (8 μmol) in 0.05 M potassium carbonate/methanol (350 μl) and acetonitrile-d_3 (50 μl), as monitored by ^{31}P NMR spectroscopy, resulting in the completely deprotected phosphate-methylated dimer d($A_{P*}T$) (**3b**). Hydrolysis of the levulinoyl ester in **3** to give **3a** (step **a**) took place within 10 minutes and is not observable in this figure.

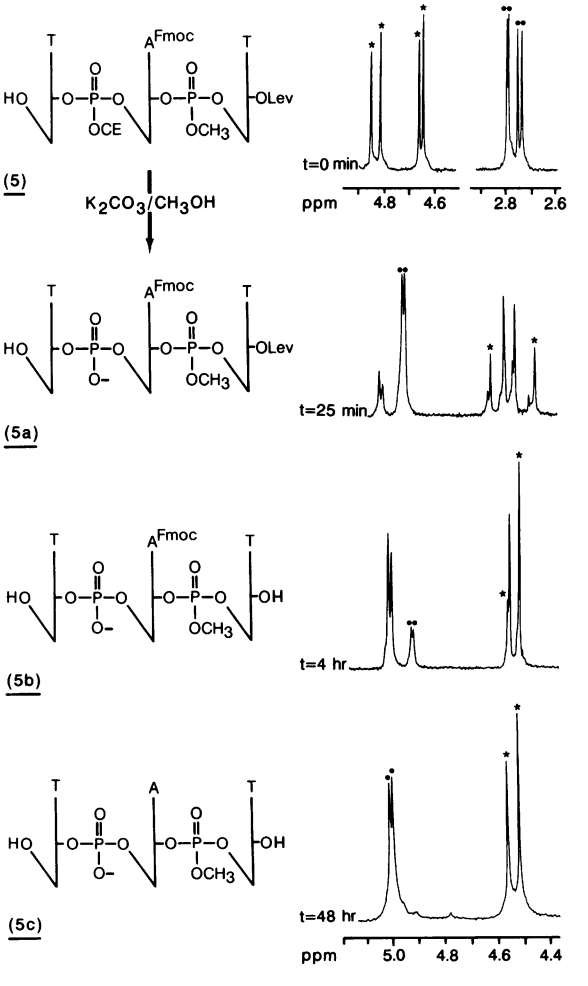


Figure 4: ^{31}P NMR monitoring of the deprotection of trimer **5** (8 μmol) in 0.05 M potassium carbonate/methanol (350 μl) and acetonitrile-d_3 (50 μl) to give **5c**. The ^{31}P NMR resonances corresponding with the methyl phosphotriester in the preceding trimer are indicated by (*). Black dots refer to the resonances of the cyanoethyl phosphotriester and phosphodiester respectively.

compound was obtained by the coupling of dimer **3** with the 5'-O-DMTr-thymidine cyanoethyl phosphoramidite (**7**), followed by oxidation, removal of the dimethoxytrityl group and silica gel chromatography. Exposure of **5** to a 0.05 M potassium carbonate solution in methanol showed, according to ^{31}P NMR observations, successively (Figure 4):

- β-Elimination of the cyanoethyl group in less then 5 minutes, leading to a phosphodiester.
- Saponification of the levulinoyl ester which reaches completion within 4 hours.
- Removal of the Fmoc group via β-elimination, affording the partially methylated trimer d(T_P-A_P*T) [1] (i.e. compound **5c**) after approximately 36 hours.

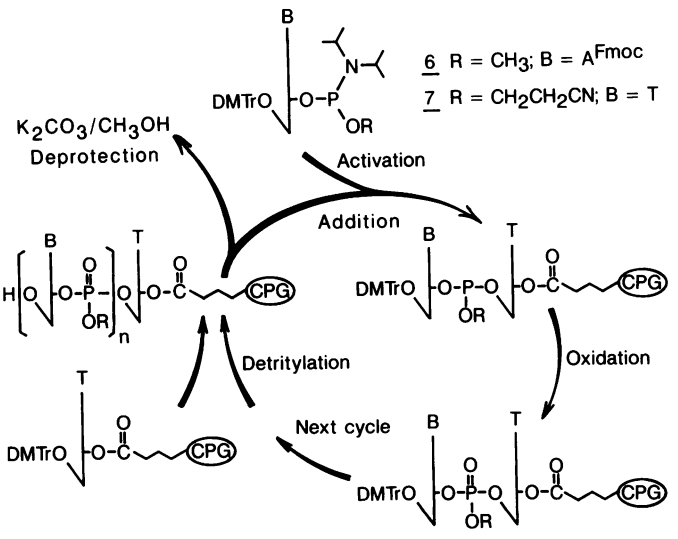
Noteworthy is the pronounced difference in required deprotection times between neutral fragments, for example **2** and **3**, on the one hand (3−4 hours) and charged systems like **5** on the other hand (36−48 hours). Fortunately, the presence of a charged phosphodiester group stabilizes the methyl phosphotriester against methanolate attack, as observed in Figure 2. Thus, when partially charged DNA fragments require a prolonged potassium carbonate/methanol treatment, no significant phosphotriester cleavage is observed.

These findings open the possibility to formulate a protocol for a solid phase synthesis of completely or partially phosphate-methylated DNA. An automatic procedure with a DNA synthesizer requires deoxynucleoside phosphoramidites, containing either a methyl or a cyanoethyl group, that are base protected with a Fmoc residue (Scheme 1).

In order to study the potassium carbonate deprotection approach in solid phase synthesis, we started with the preparation of the partially phosphate-methylated heptamer d(T_P-A_P*T_P-A_P*T_P-A_P*T). In principal, after the solid phase synthesis, the Fmoc and β-cyanoethyl groups as well as the ester linkage at the 3'-end of the fully protected heptamer can be cleaved simultaneously under conditions that have been described above for the synthesis of the partially methylated fragment d(T_P-A_P*T) (**5c**).

The fully protected heptamer was prepared on an Applied Biosystems 381A DNA synthesizer, applying a solid support of controlled pore glass loaded with 15 μmol of 5'-O-DMTr-thymidine. The methyl phosphoramidite of 5'-O-DMTr-dA-Fmoc (**6**) and the β-cyanoethyl phosphoramidite of 5'-O-DMTr-thymidine (**7**) were alternately reacted with the 5'-hydroxyl group of the DNA bound to the solid support (see Scheme 1). Since only a short sequence was being synthesized, we felt that no capping had to be performed. When all synthesis-cycles were completed, the solid support was removed from the column and treated with a freshly prepared 0.05 M solution of potassium carbonate in dry methanol (5 ml) for 43 hours at room temperature. Afterwards the reaction mixture was neutralized carefully by adding small portions of DOWEX-H^+ ion-exchange resin (during the DOWEX treatment the solution turned pale white). Next, water (5 ml) was added and the solid support and DOWEX were filtered off. Most of the methanol was removed in vacuo, resulting in a pale white aqueous phase. In order to extract fluorene and other apolar components, the aqueous phase was washed with diethyl ether. Finally, the resulting clear solution was concentrated under reduced pressure to yield a white solid. The isolated product was then analyzed by means of NMR spectroscopy and anion-exchange HPLC.

The 400 MHz ^1H NMR spectrum of the product did not reveal any Fmoc resonances, demonstrating that the applied deprotection time has been sufficient. Characteristic resonances in the ^1H NMR spectrum of the phosphate-methylated DNA fragment are the phosphate coupled methyl signals in the 4.0−3.5



Scheme 1: Solid phase synthesis cycle for the preparation of the partially phosphate-methylated heptamer d(T_P-A_P*T_P-A_P*T_P-A_P*T).

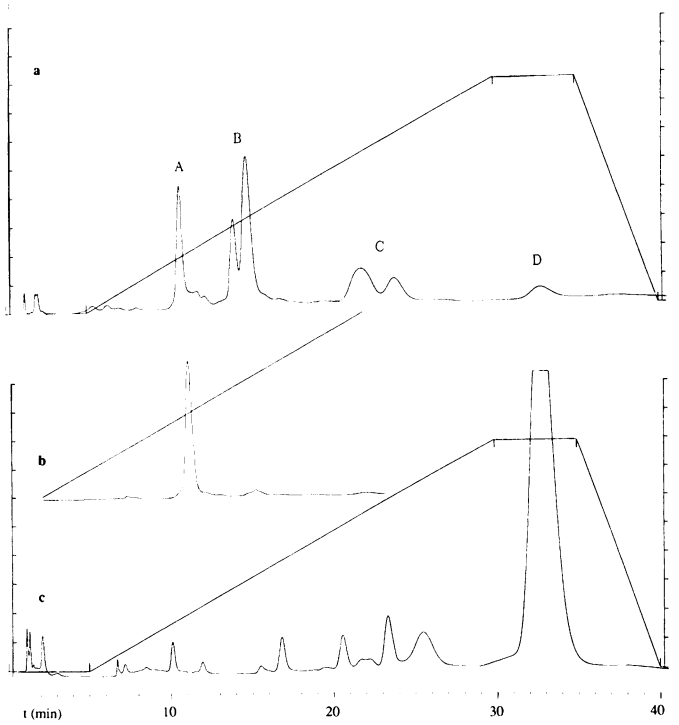


Figure 5: (a): The anion-exchange (SAX) HPLC profile of the end product in the solid phase synthesis (15 μmol, no capping included) of the partially phosphate-methylated heptamer d(T_P-A_P*T_P-A_P*T_P-A_P*T), showing four derivatives (A−D) representing heptamers with 3, 2, 1 and 0 methyl phosphotriesters respectively. (**b**): The anion-exchange (SAX) HPLC profile of fraction A in (**a**) after purification by preparative HPLC (MonoQ) and fast desalting (Superose). (c): The anion-exchange (SAX) HPLC profile obtained after treatment of the product shown in (**a**) with *tert.*-butylamine/water (50°C, 16 hr). The main peak (t = 33 min) corresponds with the completely demethylated heptamer d(T_P-A_P-T_P-A_P-T_P-A_P-T).
Conditions: Buffer A 0.02 M KH_2PO_4 (pH = 5.5, 25% acetonitrile v/v); Buffer B 0.02 M KH_2PO_4 and 1.0 M KCl (pH = 5.5, 25% acetonitrile v/v). Gradient: 0−80% B in 25 min.

ppm region. However, a comparison of the intensity of these methyl resonances with, for example, that of the $H_{1'}$, resonances, indicated that the former was too small to account for a heptamer with three methyl phosphotriesters.

Since analysis of the product by anion-exchange HPLC (SAX) showed several derivatives (A−D, Figure 5a), we focused on unravelling the side reaction that had occurred. In order to examine the possibility of chain cleavage during the potassium carbonate/methanol deprotection or during the work-up procedure, part of the product was completely demethylated by a *tert.*-butylamine/water (1:1 v/v) treatment for 16 hours at 50°C. This experiment confirmed that no chain cleavage had occurred, because HPLC analysis showed the presence of one compound, being the DNA heptamer bearing six phosphodiesters (Figure 5c).

To investigate the nature of the four major products (A−D) observed in the HPLC chromatogram, the distinct compounds were isolated by preparative anion-exchange HPLC (Mono Q), then desalted on a Superose column and lyophilized. Characterization of the four isolated fractions was performed by 400 MHz ^{1}H NMR spectroscopy. All fractions appeared to be heptamers consisting of four thymidine fragments and three adenosine residues, as may be concluded from the intensities of the resonances of the non-exchangeable base protons (H_6(T) vs. H_2(A)) and the distinct $H_{1'}$ protons ($H_{1'}$(T) vs. $H_{1'}$(A)). The ^{1}H NMR spectra of the four isolated fractions, however, clearly differ with regard to the intensity of the phosphate methyl resonances relative to the other proton resonances, (see for example $H_{5'}/H_{5''}$ of the 5'-end nucleotide, lying slightly upfield of the methyl resonances in Figure 6). In this way it was established that fraction A (see Figure 5b) consists of the desired heptamer, bearing three methyl phosphotriesters. The phosphate groups in the heptamers B, C and D were found to contain respectively 2, 1 and 0 methyl groups, in consistence with the elution pattern of these fractions in anion-exchange HPLC.

The complexity of the methyl resonances in the ^{1}H NMR spectrum of heptamer C (one methyl phosphotriester and five phosphodiesters; see Figure 6) demonstrates that the methyl group is not present on one particular phosphodiester but on either one of the three positions where methyl groups were introduced during the solid phase synthesis. These NMR results reveal that considerable demethylation has occurred, either during the solid phase synthesis or during the deprotection procedure.

In order to elucidate the stability of the partially methylated DNA fragment under the conditions of deprotection and work-up, the pure heptamer d(T_P-A_P*T_P-A_P*T_P-A_P*T) (fraction A) was treated with potassium carbonate in methanol for 16 hours. After neutralization with DOWEX-H^+ ion-exchange resin and lyophilization, the product was analyzed by anion-exchange HPLC which showed the presence of a single product, identical to the original partially methylated heptamer. Based on this result it can be concluded that the observed demethylation can not be ascribed to side-reactions during the deprotection or work-up procedure.

Fortunately, it was found that considerable improvement of the solid phase synthesis of the above mentioned heptamer can be achieved when the standard capping procedure (acetic anhydride, N-methylimidazole) between cycles is included, as illustrated in Figure 7a. Further experiments revealed that the degree of demethylation may vary among different sequences.

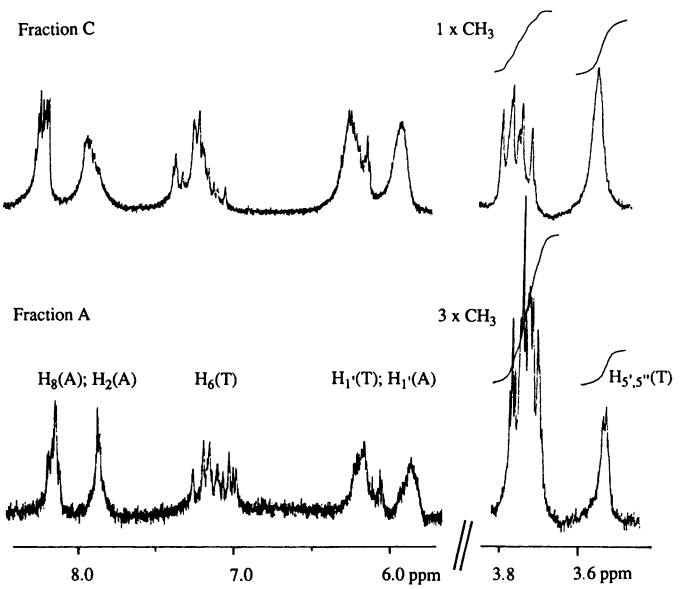


Figure 6: 400 MHz ^{1}H NMR subspectra in D_2O of the fractions A and C (Figure 5a) in the end product of the solid phase synthesis of d(T_P-A_P*T_P-A_P*T_P-A_P*T) According to the phosphate methyl resonances (3.8−3.6 ppm), lying slightly downfield of the $H_{5'}/H_{5''}$ protons of the 5'-end nucleotide (3.5 ppm), heptamers A and C contain 3 and 1 methyl phosphotriesters respectively.

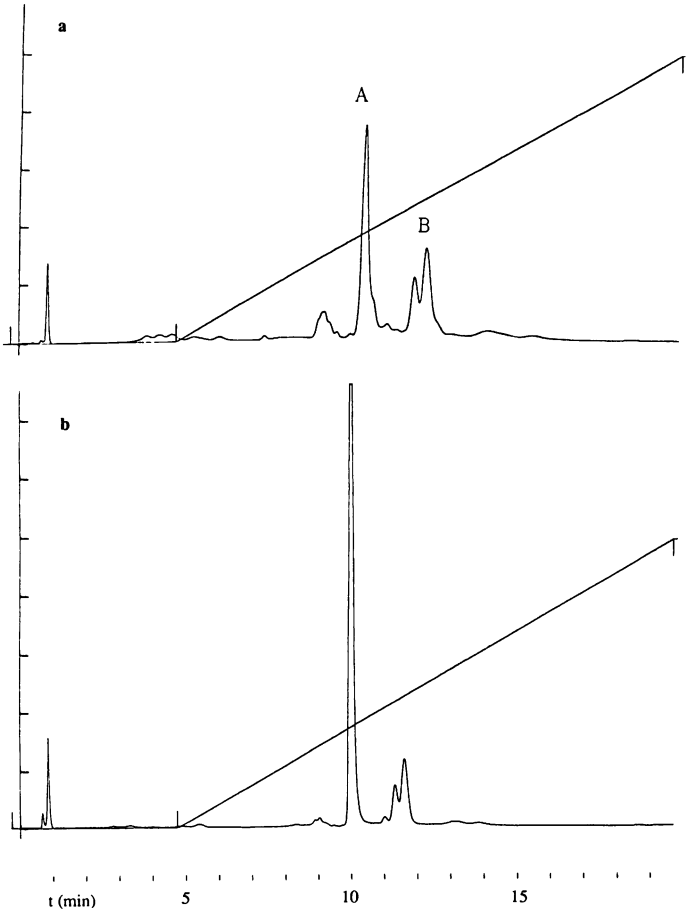


Figure 7: Anion-exchange (SAX) HPLC profiles of the end products in the solid phase synthesis (0.2 μmol) of (**a**) d(T_P-A_P*T_P-A_P*T_P-A_P*T) and (**b**) d(T_P-T_P*T_P-T_P*T_P-T_P*T) with inclusion of the standard capping procedure (acetic anhydride, N-methylimidazole).
Conditions: Buffer A 0.001 M KH_2PO_4 (pH = 6.5, 30% acetonitrile v/v); Buffer B 0.2 M KH_2PO_4 (pH = 6.5, 30% acetonitrile v/v). Gradient: 0−50% B in 15 min.

For instance, the solid phase synthesis of the partially phosphate-methylated heptamer d(T_P-T_P*T_P-T_P*T_P-T_P*T) was accompanied by less demethylation, as can be observed in Figure 7b.

CONCLUDING REMARKS

It has been demonstrated that the introduction of potassium carbonate in methanol as deprotecting reagent is a significant improvement in the solution synthesis of phosphate-methylated DNA fragments and probably also of other phosphate-alkylated nucleotides.

Regarding the application of potassium carbonate/methanol in solid phase synthesis, it was shown that this reagent could effectively remove the DNA oligomer from the CPG support and deblock the Fmoc-protected bases and cyanoethyl-protected phosphotriesters. Thus, this new methodology allows, both in solution and on a solid support, the preparation of DNA fragments which are phosphate-methylated at defined positions. One of the problems we encountered, however, is the partial demethylation of the phosphotriesters during the solid phase synthesis as observed by HPLC analysis of the end product. At the moment we are investigating at which stage of the solid phase synthesis the partial deprotection of the methyl phosphotriester occurs.

In addition, we wish to consider the introduction of base protecting groups which are more labile to methanolic potassium carbonate treatment, because the removal of the Fmoc groups turns out to be the rate-determining step in the deprotection procedure.

EXPERIMENTAL

General methods and materials

Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a Bruker AM 400 spectrometer with tetramethylsilane as internal standard. For spectra recorded in D_2O the residual HDO peak was set at 4.68 ppm. ^{31}P NMR spectra were recorded on a Bruker AM 400 spectrometer at 162 MHz with ^{1}H broad band decoupling. Chemical shifts are expressed downfield from external 85% H_3PO_4 in DO. Unless otherwise noted, reactions were run at ambient temperature and in an inert atmosphere of dry nitrogen or dry argon. Pyridine was dried by distillation from BaO. Dichloromethane was dried by distillation from potassium carbonate. Triethylamine and *tert.*-butylamine were dried by distillation from KOH pellets. 1H-Tetrazole was purified through sublimation. *tert.*-Butylhydroperoxide was used as a 75% solution in di-*tert.*-butylperoxide. The cyanoethyl phosphoramidite of 5'-O-dimethoxytrityl-thymidine (**7**) was purchased from Applied Biosystems. Merck DC-Fertigplatten Kieselgel 60 F_{254} were used for TLC analysis. All column chromatographic separations were performed on Kieselgel 60, 70–230 mesh ASTM.

Methanol, used for the deprotection experiments, was dried by refluxing on magnesium turnings for 2 hours and then distilled and stored over molecular sieves (3 Å). Potassium carbonate was dried in vacuo (P_2O_5) for 72 hours at 40°C. A 0.05 M potassium carbonate solution was prepared by adding dry methanol to the required amount of potassium carbonate and subsequently sonified for 45 minutes under an argon atmosphere to give a clear solution. DOWEX 50X8–100 ion-exchange resin, used for neutralization in the deprotection experiments, was thoroughly washed with water and dry methanol before use. DOWEX-Na^+ cation-exchange resin was prepared from DOWEX 50X8–100 by treatment with 1 N NaCl and 1 N NaOH successively.

High-performance liquid chromatography (HPLC) analysis was carried out on a LKB system consisting of two 2248 HPLC-pumps and a 2252 LC controller. UV-absorption at 254 nm, was monitored with a 2510 Uvicord detector. Analytical anion-exchange HPLC was performed on a self-packed Zorbax SAX (7.5 μm) column, 100×4.0 mm; flowrate 1.0 ml/min. Elution conditions (**I**)[28] and (**II**)[29] appeared suitable for SAX-HPLC. (**I**): Buffer A 0.02 M KH_2PO_4 (pH = 5.5, 25% acetonitrile v/v); Buffer B 0.02 M KH_2PO_4 and 1.0 M KCl (pH = 5.5, 25% acetonitrile v/v); gradient: 0–5 min isocratic 100% A, 5–30 min linear 0–80% B. (**II**): Buffer A 0.001 M KH_2PO_4 (pH = 6.5, 30% acetonitrile v/v); Buffer B 0.2 M KH_2PO_4 (pH = 6.5, 30% acetonitrile v/v); gradient: 0–5 min isocratic 100% A, 5–20 min linear 0–50% B.

Preparative anion-exchange HPLC was carried out on a Waters 600 E (system controller) single-pump gradient system, equipped with a Waters model 484 variable wavelength UV-detector and a Waters model 741 data module under the following conditions: Pharmacia FPLC ion-exchange column MonoQ HR 10/10; eluent system (**I**); gradient 0–5 min isocratic 100% A, 5–30 min linear 0–60% B; detection 254 nm; flowrate 3.0 ml/min. Fast desalting was performed on a Pharmacia FPLC Sephadex G-25 superfine column HR 10/16, using water (milliQ) as eluent; flowrate 2.0 ml/min.

Preparation of compounds 1–5

The phosphate-methylated dimers **1**–**3** were prepared according to described literature procedures, involving, amongst others:

—protection of the base amino groups with Fmoc via transient protection of the 5'- and 3'-hydroxyl functions with chlorotrimethylsilane.[25,30]

—coupling of two appropriately protected nucleosides via an *in situ* generated phosphoramidite synthon.[25,31]

—removal of the levulinoyl protective group[32] through treatment with hydrazine hydrate in pyridine-acetic acid.[33]

The fully protected dinucleotide **4** was synthesized by reacting the cyanoethyl phosphoramidite of 5'-O-DMTr-T (**7**) with 3'-O-Levulinoyl-thymidine in the presence of 1H-tetrazole, followed by oxidation with *tert.*-butylhydroperoxide.

Preparation of trimer **5**

Compound **3** (0.45 g, 0.51 mmol) was dissolved in anhydrous dichloromethane (2 ml) and 1H-tetrazole (5 ml of a 0.5 M solution in acetonitrile, 2.5 mmol) was added. This solution was added to a solution of the cyanoethyl phosphoramidite of 5'-O-dimethoxytrityl-thymidine (**7**) in anhydrous dichloromethane (2 ml). After 30 minutes ^{31}P NMR analysis revealed the complete conversion of the phosphoramidite into a phosphite triester, which was readily oxidized through the addition of *tert.*-butylhydroperoxide (0.25 ml). After 15 minutes anhydrous dichloromethane (50 ml) was added and the solution was washed with 5% aqueous sodium hydrogen carbonate solution (2×25 ml) and water (25 ml). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure, affording a white solid (0.95 g). The fully protected trimer (R_f: 0.46 ($CHCl_3$-CH_3OH 9:1 v/v)) was detritylated without further purification by dissolving the solid in dichloromethane-methanol (9:1 v/v, 10 ml) and adding dropwise dichloroacetic aid (0.5 ml). After 30 minutes the reaction mixture was diluted with dichloromethane-

methanol (9:1 v/v, 10 ml) and 5% aqueous sodium hydrogen carbonate (25 ml) was added. The organic layer was washed with 5% aqueous sodium hydrogen carbonate (25 ml) and water (25 ml), next, dried ($MgSO_4$) and concentrated to a small volume (2 ml). Methanol (5 ml) was added and the resulting solution was evaporated under reduced pressure. The obtained white solid was fractionated by column chromatography on silica gel using a gradient of methanol in dichloromethane (4 → 12 vol% ethanol). The appropriate fractions were combined and concentrated under reduced pressure, yielding a white solid (0.49 g, 76%). R_f (**5**): 0.17 ($CHCl_3$-CH_3OH 9:1 v/v).

^{31}P NMR ($CDCl_3$): δ(P-OCH_3): −0.14, −0.16 and −0.32 ppm (intensities 1:1:2) δ(P-OCE): −1.96, −2.14 and −2.22 ppm (intensities 2:1:1).

^1H NMR ($CDCl_3$) of a mixture of diastereoisomers: δ 8.78 (1H,s,H_8(A)), 8.40 (1H,s,H_2(A)), 7.73 (3H,m,H_6(T) and aromatic Fmoc), 7.51 (1H,s,H_6(T)), 6.52 (1H,dd,$H_{1'}$), 6.29 (1H,dd,$H_{1'}$), 6.22 (1H,dd,$H_{1'}$), 5.33 (1H,m,$H_{3'}$), 5.27 (1H,m,$H_{3'}$), 5.12 (1H,m,$H^{3'}$), 3.85 (3H, 4×d, P-OCH_3), 3.78 (2H,m,$H_{5'}$/$H_{5''}$(T)), 2.70 and 2.47 (2H, 2×t, CH_2-CN), 2.16 and 2.12 (3H, 2×s, CH_3 Lev), 1.92 (3H,s,-CH_3(T)), 1.82 (3H,s,CH_3(T)).

Stability experiments with DMTr-T-$_P$*-T-OH (1) (Figure 2)

To DMTr-T-$_P$*-T-OH (6.9 mg, 8 μmol) was added anhydrous pyridine (100 μl), anhydrous pyridine-d_5 (100 μl) and anhydrous triethylamine (200 μl). The stability of the methyl phosphotriester was monitored by ^{31}P NMR spectroscopy.

δ(**1**): 0.51 and 0.43 ppm

δ(demethylated dimer): −0.29 ppm

DMTr-T-$_P$*-T-OH (6.9 mg, 8 μmol) was dissolved in a freshly prepared 0.05 M potassium carbonate solution in anhydrous ethanol (350 μl). Acetonitrile-d_3 (50 μl) was added and ^{31}P NMR was performed to determine the stability of the methyl phosphotriester.

δ(**1**): 4.83 and 4.73 ppm.

During the experiment trace amounts of side-products were observed at 6.90, 5.84 and 5.05 ppm.

Deprotection experiments in 0.05 M potassium carbonate in methanol

Deprotection of the Fmoc-protected 5'-O-Dimethoxytrityl-deoxynucleosides (**9a−9c**).

0.05 M Potassium carbonate in methanol (500 μl) was added to the Fmoc-protected deoxynucleoside (10 μmol). The deprotection reaction was monitored by TLC analysis ($CHCl_3$-CH_3OH 9:1 v/v).

R_f DMTr-dA^{Fmoc} : 0.59 R_f DMTr-dA : 0.32 Deprotection complete in 3 hours.

R_f DMTr-dG^{Fmoc} : 0.40 R_f DMTr-dG : 0.07 Deprotection complete in 4 hours.

R_f DMTr-dC^{Fmoc} : 0.54 R_f DMTr-dC : 0.18 Deprotection complete in 4 hours.

(DMTr-dC^{Fmoc} is not completely soluble in potassium carbonate-methanol. The reaction mixture becomes clear during the deprotection).

Compound		δ ^{31}P P(OCE)/P(O-) (ppm)	δ ^{31}P P* (ppm)
HO-T-$_{P(OCE)}$-dA^{Fmoc}-$_P$*-T-OLev	(**5**)	2.74, 2.76, 2.79, 2.80 #	4.65, 4.67, 4.82, 4.85 #
HO-T-$_{P(O-)}$-dA^{Fmoc}-$_P$*-T-OLev	(**5a**)	4.95, 4.96	4.45, 4.62
HO-T-$_{P(O-)}$-dA^{Fmoc}-$_P$*-T-OH	(**5b**)	4.95, 4.96	4.52, 4.57
HO-T-$_{P(O-)}$-dA-$_P$*-T-OH	(**5c**)	5.02, 5.04	4.52, 4.56

#: in methanol (350 μl) and acetonitrile-d_3(50 μl).

Deprotection of DMTr-dA^{Fmoc}-$_P$*-T-OLev (**2**) *in potassium carbonate-methanol.*

Compound **2** (8.3 mg, 7 μmol) was dissolved in a freshly prepared 0.05 M potassium carbonate solution in anhydrous methanol (350 μl) and, subsequently, anhydrous acetonitrile-d_3 (50 μl) was added. Cleavage of the Fmoc protective group and hydrolysis of the levulinoyl ester were monitored by TLC analysis ($CHCl_3$-CH_3OH, 85:15 v/v) and ^{31}P NMR measurements.

The stability of the methyl phosphotriester was determined by ^{31}P NMR spectroscopy. Trace amounts of side-products were observed at 6.90, 5.84 and 5.05 ppm during the deprotection reaction (Figure 2).

Deprotection of HO-dA^{Fmoc}-$_P$*-T-OLev (**3**) *in potassium carbonate-methanol.* (Figure 3)

Dimer **3** (35.6 g, 40 μmol) was dissolved in 0.05 M potassium carbonate in methanol (2.0 ml). A small quantity (350 μl) of this solution was transferred to a NMR sample tube filled with acetonitrile-d_3 (50 μl). The deprotection reaction was monitored by TLC and ^{31}P NMR analysis.

Determination of the stability of the methyl phosphotriester was performed by ^{31}P NMR measurements (side-products at 6.90, 5.84 and 5.19 ppm) (Figure 2).

After a deprotection time of 6 hours, the solution was diluted with anhydrous methanol (3.5 ml) and neutralized by adding small portions of DOWEX-H^+ ion-exchange resin. The ion-exchange resin was filtered off and washed with anhydrous methanol (2 ml) and water (2×4 ml). The filtrate was concentrated to a small volume (2 ml) under reduced pressure, resulting in a pale white aqueous phase. After addition of water (2 ml) and methanol (2 ml), the aqueous phase was washed with diethyl ether (3×6 ml) and, subsequently, evaporated under reduced pressure to give a white solid. After coevaporation with D_2O (2×2 ml), a NMR sample was prepared by addition of D_2O (600 μl) and, in order to completely dissolve the product, acetonitrile-d_3 (100 μl). ^{31}P NMR (**3b**) (D_2O/CD_3CN 6:1 v/v): δ 2.23 and 2.09 ppm. ^1H NMR (**3b**) (D_2O/CD_3CN 6:1 v/v): δ 8.22 (1H,s,H_8(A)), 8.16 (1H,s,H_2(A)), 7.42 (1H,s,H_6(T)), 6.39 (1H,dd,$H_{1'}$(A)), 6.21 (1H,dd,$H_{1'}$(T)), 5.19 (1H,m,$H_{3'}$(A)), 3.87 and 3.86 (3H,2×d,P-OCH_3), 1.81 and 1.75 (3H,2×s,CH_3-(T)).

Deprotection of DMTr-T-$_{P(OCE)}$-T-OLev (**4**) *in potassium carbonate-methanol.*

A freshly prepared 0.05 M solution of potassium carbonate in methanol-d_4 (1.0 ml) was added to compound **4** (20 g, 20 μmol). TLC analysis revealed cleavage of the β-cyanoethyl group within 5 minutes (R_f: 0.58 → 0 $CHCl_3$-CH_3OH 85:15 v/v). The hydrolysis of the levulinoyl ester was monitored by ^{31}P NMR spectroscopy ($t_{1/2}$ = 30 min): δ(DMTr-T-$_{P(O-)}$-T-OLev): 3.60 ppm δ(DMTr-T-$_{P(O-)}$-T-OH): 3.84 ppm.

Deprotection of trimer **5** *in potassium carbonate-methanol.* (Figure 4)

Compound **5** (60 mg, 48 μmol) was dissolved in a freshly prepared 0.05 M solution of potassium carbonate in methanol (2.1 ml) by sonification during 10 minutes. A small quantity (350 μl) of this solution was added to a NMR sample tube with anhydrous acetonitrile-d_3 (50 μl) in order to monitor the deprotection reaction with ^{31}P NMR spectroscopy.

After 48 hours, when the deprotection had gone to completion, the solution was diluted with anhydrous methanol (5 ml) and neutralized by addition of small portions of DOWEX-H^+ ion-

exchange resin. After addition of water (10 ml), the resin was removed by filtration and washed with methanol (5 ml), and water (10 ml). The filtrate was concentrated to 10 ml and washed with diethyl ether (3×10 ml). The resulting solution was concentrated to a small volume (1 ml), brought onto a column of DOWEX-Na^+ cation-exchange resin and eluted with water. Finally, the solution was evaporated under reduced pressure to afford the deprotected trimer **5c** as a white solid.

^{31}P NMR (D_2O): δ 1.92, 1.87, 1.84 and 1.83 ppm. ^1H NMR (D_2O) of a mixture of diastereoisomers: δ 8.31 and 8.28 (1H,2×s,H_8(A)), 8.02 (1H,s,H_2(A)), 7.39 (1H,s,H_6(T)), 7.19 (1H,s,H_6(T)), 6.25 (1H,dd,$H_{1'}$), 6.12 and 6.03 (1H,2×dd,$H_{1'}$), 5.87 (1H,dd,$H_{1'}$), 5.23 (1H,m,$H_{3'}$(A)), 3.79 (3H,2×d,P-OCH_3), 3.55 (2H,m,$H_{5'}/H_{5''}$(T)), 1.72 (3H,s,CH_3(T)), 1.67 and 1.62 (3H,2×s,CH_3(T)).

5'-O-(4,4'-Dimethoxytrityl)-6-N-(9-fluorenylmethoxycarbonyl)-2'-deoxyadenosine-3'-O-methyl-N,N-diisopropylphosphoramidite (6)

The 3'-phosphoramidite **6** was synthesized by phosphitylation of DMTr-dA^{Fmoc} (**9a**) using chloro-N,N-diisopropylamino methoxyphosphine, essentially as previously described.[34]

The nucleoside derivative **9a** (3.9 g, 5.0 mmol) was dissolved in anhydrous dichloromethane (10 ml) and N,N-diisopropylethylamine (3.3 l, 15 mmol) was added. The solution was cooled in an ice-water bath and chloro-N,N-diisopropylamino methoxyphosphine (1.4 ml, 7.5 mmol) was then added dropwise with stirring over a period of 30 seconds. After stirring at room temperature for 45 minutes, TLC (dichloromethane/ethylacetate/2,6-lutidine 50:50:2 v/v/v) showed the complete conversion of **9a** into higher R_f products. Excess phosphitylating reagent was quenched by adding anhydrous methanol (0.1 ml). The mixture was diluted with ethylacetate (100 ml) and washed with ice-cold saturated aqueous sodium hydrogen carbonate solution (2×100 ml). The dried ($MgSO_4$) ethylacetate layer was concentrated under reduced pressure. A solution of the residue in toluene (25 ml) was added dropwise with stirring to cold (−20°C) hexane (500 ml) to give the desired amidite as a white powder (4.0 g, 85%). ^{31}P NMR analysis of the product revealed the presence of two diastereoisomeric phosphoramidites, while no side-products were detected. Therefore, the amidite **6** was used for solid phase synthesis without further purification. R_f (**6**) (dichloromethane/ethylacetate/2,6-lutidine 50:50:2 v/v/v) 0.49 and 0.56.

^{31}P NMR ($CDCl_3$): δ 149.8 and 149.6 ppm.

^1H NMR ($CDCl_3$): δ 8.70 (1H,s,H_8), 8.39 (1H,bs,NH Fmoc), 8.16 and 8.14 (1H,s,H_2), 7.8−7.6 (4H,2×d, aromatic Fmoc), 7.45−7.15 (13H,m,aromatic Fmoc and DMTr), 6.78 (4H,2×d, aromatic DMTr), 6.48 (1H,dd,$H_{1'}$), 4.75 (1H,m,$H_{3'}$), 4.61 (2H,d,CH_2 Fmoc), 4.32 (1H,m,$H_{4'}$), 4.31 (1H,t,CH Fmoc), 3.76 (6H,s,OCH_3 DMTr), 3.58 (2H,m,CH iPr), 3.40 and 3.32 (3H,2×d, OCH_3; J_{POCH3} = 13.3 Hz), 3.36 (2H,m,$H_{5'}/H_{5''}$), 2.86 (1H,m,$H_{2'}$), 2.65 (1H,m,$H_{2''}$).

Using the same protocol we succeeded in the preparation of the methyl phosphoramidites of DMTr-dC^{Fmoc} and DMTr-dG^{Fmoc}. Furthermore, by applying chloro-N,N-diisopropylamino 2-cyanoethoxyphosphine as phosphitylating reagent the β-cyanoethyl phosphoramidites of the three Fmoc-protected deoxynucleosides were synthesized.

Solid phase synthesis of the partially phosphate-methylated heptamer d(T_P-A_P*T_P-A_P*T_P-A_P*T)

The fully protected heptamer was synthesized on an Applied Biosystems Synthesizer model 381A, employing the cycle described in Scheme 1. The synthesis was carried out using a column with 15 μmol immobilized 5'-O-DMTr-thymidine on a 500 Å CPG support. The commercially available cyanoethyl phosphoramidite of 5'-O-DMTr-thymidine (**7**) and the methyl phosphoramidite of 5'-O-DMTr-dA^{Fmoc} (**6**) were applied as 0.1 M solutions in anhydrous acetonitrile (DNA synthesis grade). When the solid phase synthesis was completed, the CPG support was removed from the column and treated with 0.05 M potassium carbonate solution in anhydrous methanol (5 ml) during 43 hours at room temperature. Next, the clear solution was neutralized by adding small portions of DOWEX-H^+ ion-exchange resin (washed first with anhydrous methanol). During the neutralization the solution turned pale white. After addition of water (5 ml), affording a clear solution, the CPG and ion-exchange resin were filtered off and washed thoroughly with water (3×5 ml) and ethanol (2×5 ml). The filtrate was concentrated to a small volume (5 ml) under reduced pressure, resulting in a pale white suspension. The aqueous phase was washed with diethyl ether (3×6 ml) and evaporated under reduced pressure. The residue was eluted with water on a column of DOWEX-Na^+ cation-exchange resin. The solution containing DNA was concentrated under reduced pressure and dissolved in water (2 ml).

A small quantity of this solution (10 μl) was diluted with water-acetonitrile (3:1 v/v, 100 μl) and analyzed by HPLC-SAX anion-exchange chromatography (eluent conditions (**I**), see general methods) (Figure 5a).

Additionally, a volume of the above mentioned DNA solution (250 μl) was diluted with water (250 μl) and treated with *tert.*-butylamine (500 μl) at 45°C during 16 hours, in order to achieve complete demethylation of the DNA. Subsequently, *tert.*-butylamine was evaporated under reduced pressure and the residue was coevaporated with water (2×1 ml). The obtained white solid was dissolved in water-acetonitrile (3:1 v/v, 1 ml) and analyzed by HPLC-SAX (Figure 5c).

The remaining DNA solution was concentrated under reduced pressure and the residue was dissolved in water-acetonitrile (3:1 v/v 600 μl). The four distinguished derivatives (A−D, Figure 5a) were isolated by preparative HPLC-MonoQ anion-exchange chromatography (eluent conditions (**I**), see general methods) in four runs. The corresponding fractions were combined, concentrated to a small volume (2 ml) and desalted on a Superose column using water as eluent (see general methods). Next, the four fractions were lyophilized twice with D_2O and, finally, dissolved in D_2O (400 μl).

^{31}P NMR (Fractions A−D) (D_2O): δ 2.0−1.7 ppm. ^1H NMR (Fractions A−D) (D_2O): δ 8.2 (3H,H_8(A)), 7.9 (3H,H_2(A)), 7.15 (4H,H_6(T)), 6.15 (4H,$H_{1'}$(T)), 5.8 (3H,$H_{1'}$(A)), 5.2 (var.,$H_{3'}$-(A_P-OCH_3)), 3.75 (va$_P$.,P-OCH_3), 3.5 (2H,$H_{5'}/H_{5''}$(5'-T)), 2.7 (7H,$H_{2'}$(A) and $H_{2'}$(T)), 2.1 (7H,$H_{2''}$(A) and $H_{2''}$(T)), 1.6 (12H,CH_3(T)).

The 0.2 μmol solid phase synthesis of the heptamers d(T_P-A_P*T_P-A_P*T_P-A_P*T) and d(T_P-T_P*T_P-T_P*T_P-T_P*T) were carried out as described above. In contrast to the 15 μmol synthesis of d(T_P-A_P*T_P-A_P*T_P-A_P*T), after each coupling step a capping procedure (acetic anhydride and N-methylimidazole,

10 seconds per cycle) was performed. The fully protected heptamers were treated with 0.05 M potassium carbonate in anhydrous methanol (0.5 ml) and after a deprotection time of 4 hours (d(T_P-T_P*T_P-T_P*T_P-T_P*T)) and 48 hours (d(T_P-A_P*T_P-A_P*T_P-A_P*T)) respectively, anhydrous methanol (0.5 ml) was added. The reaction mixture was neutralized by adding small portions of DOWEX-H^+ ion-exchange resin. Next, water (1.0 ml) was added and the CPG and ion-exchange resin were filtered off and thoroughly washed with methanol and water. The solution was concentrated to a small volume (1 ml) and washed with diethyl ether (3×2 ml) before HPLC (SAX) analysis.

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